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SMALL PEPTIDES AND METHODS FOR DOWNREGULATION OF IgE

FIELD OF THE INVENTION

This invention relates to small peptides, particularly to N-formyl-methionyl peptides, having downregulating activity of IgE and to methods for treating indications resulting from IgE-mediated responses. More particularly, the peptides can be used to replace corticosteroids in any application in which corticosteroids are used.

BACKGROUND OF THE INVENTION

Immunoglobulin E (IgE) is one of five classes of antibody occurring in man and has been known for over three decades that it is the immunoglobulin responsible for allergic reactions. IgE is produced and secreted by B cells upon allergen invasion. However, IgE constitutes only a small fraction of the total antibody in human serum (50-300ng/ml compared to 10mg/ml of IgG) and thus, is not present in sufficient amount to directly neutralize antigens. Instead, its action is amplified through target cellular receptors and elicits a wide range of cellular responses to antigens, culminating in inflammation, itching, coughing, lacrimation, bronchoconstriction, mucus secretion, vomiting and diarrhea, all symptoms commonly associated with allergic disorders.

Immediate hypersensitivity reactions are triggered through the high-affinity IgE receptors ($Fc_\epsilon RI$) found on mast cells and basophils. Allergen binding to the $Fc_\epsilon RI$-bound IgE causes cross-linking of receptor molecules on the cell membrane, which triggers degranulation of the cell and subsequent release of histamines and other mediators associated with the immediate phase of the allergic response. These products of mast cell degranulation cause activation of inflammatory cells and further induces a low-affinity IgE receptor, $Fc_\epsilon RII$, also known as CD23. $Fc_\epsilon RII$ can be found

on activated B cells, various inflammatory cells (macrophages, eosinophils, platelets, natural killer cells), T cells, follicular dendritic cells (FDC), Langerhans cells and epithelial cells of the bone marrow and thymus (Delespesse et al., *Adv. Immun.* **49**:149-190, 1991; Delespesse et al., *Immunol. Rev.* **125**:78-97, 1992). On the surface of B cells, $Fc_{\varepsilon}RII$ plays a role in IgE-dependent antigen presentation to T cells and also in the cross-linking of B cells. On FDCs, $Fc_{\varepsilon}RII$ is expressed in large amounts and is therefore implicated in the recruitment of B cells to the germinal centers of secondary follicles in the lymph nodes and spleen. When expressed on inflammatory cells, it is thought to be responsible for IgE-dependent cytotoxic activities, such as phagocytosis of immune complexes by monocytes. Soluble $Fc_{\varepsilon}RII$ ($sFc_{\varepsilon}RII$) can also initiate humoral and cell-mediated immune responses by triggering the growth and differentiation of precursors of plasma cells, T cells and basophils.

Differentiation of B cells into IgE-secreting plasma cells involves a complex signaling cascade of cytokines and surface molecules, thought to take place mainly in the germinal centers of secondary follicles in the lymph nodes and spleen. Surface molecules are essential in order to provide the physical interaction of B cells with T cells and mast cells that is required for triggering IgE production. These surface molecules are CD40 ligand (CD40-L) and $Fc_{\varepsilon}RII$. When T helper type 2 cells (Th_2) are activated upon exposure to antigen-presenting cells (APCs), they transiently express CD40L. CD40L interacts with CD40 on B cells, resulting in B cell activation. The activated Th_2 cells secrete various cytokines, such as IL-4 and IL-13, which act on the activated B cells to switch to IgE production. IL-4 in addition upregulates $Fc_{\varepsilon}RII$ expression on B cells and inflammatory cells, providing a further source of contact stimulation and soluble growth factor.

Mast cells and basophils also secrete IL-4 and express CD40L and can thus induce IgE synthesis by B cells upon physical interaction with B cells in the presence of IL-4, in a similar manner as Th_2 cells. It is likely that IgE synthesis can also take place in the skin, lungs and gut, in view of the tissue distribution of the various types of cells involved in IgE production.

Upregulation of IgE synthesis and rescue of germinal center B cells from apoptosis is mediated by the cross-linking of B cell membrane-bound IgE and complement receptor 2 (CR2), also called CD21, by $sFc_\epsilon RII$. CR2 is a highly glycosylated membrane protein found on B cells, FDCs, and some T cells and basophils. $sFc_\epsilon RII$ can participate in the positive feedback control of IgE synthesis by triggering CR2 on B cells to enhance IgE synthesis while also promoting the survival of IgE-committed B cells.

Activation of IgE production can lead to two different situations. Acute inflammation due to allergen exposure begins with an early phase reaction involving rapid activation of mast cells, airway macrophages, and bronchial epithelial cells which release proinflammatory mediators including histamines, eicosanoids, platelet-activating factor, oxygen free radicals, neuropeptides, and cytokines. These can induce constriction of the airway smooth muscle, mucous secretion, and vasodilation. Inflammation of the airways causes increased microvascular leakage, leading to plasma exudation into the airways. Thickening of airway walls and narrowing of the airway lumen result.

In the late-phase reaction, peripheral blood cells are recruited into the airways to establish a chronic-type of inflammation. Such cells include eosinophils, lymphocytes, and monocytes, and recruitment is dependent on cytokines such as IL-5 and granulocyte-macrohage colony-stimulating factor (GMC-SF). Chemokines such as RANTES and eotaxin also appear to

enhance recruitment of eosinophils. At the site of inflammation, these cells are activated and their survival is increased by reduced apoptosis, mediated by factors such as GMC-SF.

Treatments for asthma have traditionally been based on the severity and persistence of the disorder. For acute, intermittent symptoms, treatments have generally involved bronchodilators. Bronchodilators include β-adrenergic agonists, methylxanthines, and anticholinergic drugs. These agents can improve airway obstruction in asthma patients but they do not appear to be effective in reducing airway inflammation or bronchial hyperreactivity. In more recent years, leukotriene inhibitors have become available for treatment of mild to moderate asthma. Leukotrines are generated from arachidonic acid through the 5-lipoxygenase metabolic pathway and have long been known to possess powerful bronchoconstrictive properties. These so-called slow-reacting substance of anaphylaxis ("SRS-A") also induce migration, adhesion and aggregation of various white blood cells to blood vessels and increase capillary permeability, culminating in interstitial edema, leukocyte chemotaxis, mucus production, mucocilliary dysfunction, and bronchospasm in the lungs. Leukotriene D_4 (LTD_4), in particular, appears to be primarily responsible for this activity in airways and acts through a specific receptor on airway smooth muscle cells. Leukotrines, including cysteinyl leukotrienes, are released during IgE-mediated mast cell degranulation.

Leukotriene inhibitors consist of two types: one that blocks the synthesis of leukotrienes by inhibiting the activity of 5-lipoxygenase (5-LO), which is required for the synthesis of leukotriene, and another that competitively blocks the LTD_4 receptor on smooth muscle cells. Zileutin is the first of the 5-LO inhibitors that have become available. Zafirlukast is the first LTD_4 receptor antagonist to be approved, while others such as monelukast and pranlukast are currently undergoing clinical trials. These

leukotriene inhibitors have so far been used for treatments of mild persistent asthma but have not yet been proved effective for more severe forms of asthma.

Antiinflammatory agents are currently employed for treating more severe and persistent forms of asthma. Agents categorized as antiinflammatory agents include theophylline, corticosteroids, cromolyn sodium, and nedocromil sodium. Corticosteroids, in particular, appear to be more effective in decreasing bronchial hyperreactivity and severe exacerbations. They act by suppressing eosinophil recruitment by inhibiting cytokine and chemokine production, as well as by inducing apoptosis of eosinophils. They also act to abrogate airway edema and bronchorrhea and therefore, inhaled corticosteroids are the most common treatment for patients with chronic asthma. Inhaled corticosteroids include beclomethasone, flunisolide, triamcinolone, fluticasone, and budesonide. For chronic asthma, β_2-agonists are ineffective, except in that they can temporarily improve bronchial obstruction. Thus, optimal treatment may be to combine both inhaled corticosteroids and long-acting β_2-agonists. However, potential side effects of corticosteroids include oropharyngeal candidiasis, dysphonia, adrenal suppression, growth retardation in children, thinning of skin, osteoporosis, glaucoma, and cataracts. In addition, it is unclear at the present time, the relationship between "effective" versus "toxic" doses of these corticosteroids.

In addition to targeting the downstream events of the IgE signaling pathway, some new therapeutic strategies are being developed to directly intervene with IgE and its synthesis. The central position IgE plays in the complex network leading to allergic reactions suggests that therapy targeted to eliminate IgE or to block IgE binding to receptors would in effect, prevent allergic responses altogether. Although still in its early stages, some success has been shown by the use of monoclonal antibodies directed

against IgE. Fahy et al. (*Am. J. Respir. Crit. Care Med.*, **155**:1828-1834, 1997) have reported that a humanized murine monoclonal antibody developed against IgE reduced free IgE and was successful in blocking both the early and late phase responses to allergen stimulation. Anti-IgE antibodies that target a region of IgE necessary for binding to $Fc_\epsilon RI$ not only blocks binding of IgE to its receptor, but also prevents mast cell degranulation and anaphylaxis induced by the cross-linking of IgE bound to $Fc_\epsilon RI$ on basophils and mast cells. Two such anti-IgE antibodies are currently being tested (MacGlashan et al., *J Immunol.* **158**:1438-1445, 1997; Corne et al., *J. Clin. Invest.* **99**:879-887, 1997). So far, they appear to reduce IgE concentrations in serum and also lower the levels of $Fc_\epsilon RI$ on basophils, suggesting that IgE-dependent responses may be altered by modulating the levels of circulating IgE.

The treatments to date typically have focused on downstream events, which result from IgE activation. It would therefore be desirable to develop treatments that modulate IgE levels in order to treat IgE-mediated responses. Chemotactic peptides such as N-formyl-methionyl-leucyl-phenylalanine and pepstatin have been reported to inhibit mast cell degranulation (*Inflammation*, Vol. 5, No. 1, pp. 13-16, 1981). The peptides of the present invention downregulate IgE levels and therefore can be used to modulate a variety of IgE-mediated responses.

SUMMARY OF THE INVENTION

The present invention provides methods for treating a variety of indications resulting from IgE-mediated responses using pharmaceutical compositions containing in a suitable pharmacological carrier a N-formyl-methionyl-leucyl ("f-Met-Leu") peptide having IgE-downregulation activity. Particularly useful peptides are those having the formula f-Met-Leu-X where X is selected from the group consisting of Tyr, Tyr-Phe, Phe-Phe and Phe-

Tyr. The peptides of the present invention can be used to replace corticosteroids in any application in which corticosteroids are used.

In accord with the present invention, a method for treating an IgE-mediated response in a mammal comprises administering to the mammal an IgE downregulating effective amount of a peptide having the formula f-Met-Leu-X where X is selected from the group consisting of Tyr, Tyr-Phe, Phe-Phe and Phe-Tyr.

The invention also provides a method for downregulating membrane-bound and soluble receptors for IgE. The method comprises administering to the patient a IgE receptor downregulating effective amount of a peptide having the formula f-Met-Leu-X where X is selected from the group consisting of Tyr, Tyr-Phe, Phe-Phe and Phe-Tyr.

The invention further provides a method for inhibiting IgE secretion by plasma cells. The method comprises administering to the patient an IgE secretion inhibiting effective amount of a peptide having the formula f-Met-Leu-X where X is selected from the group consisting of Tyr, Tyr-Phe, Phe-Phe and Phe-Tyr.

In accord with another embodiment, the invention provides a method for downregulating CD40L expression. The method comprises administering to a patient a CD40L downregulating effective amount of a peptide having the formula f-Met-Leu-X where X is selected from the group consisting of Tyr, Tyr-Phe, Phe-Phe and Phe-Tyr.

In certain preferred embodiments of the present invention, patients can benefit by administering the peptide of the present invention in combination with a second active ingredient. Particularly useful other active ingredients for such combination in accord with the present

invention are, for example, antileukotrienes, beta$_2$ agonists, corticosteroids, and the like.

BRIEF DESCRIPTION OF DRAWINGS

FIG. 1 is a log dose response curve illustrating the effects of various dosages of HK-X on OVA-specific serum IgE levels in acute asthmatic mice.

FIG. 2 shows lung sections from acute asthmatic mice administered with 50 μg of HK-X. Limited cellular infiltrates were present in (A) and (B) and limited mucus accumulation in (C).

FIG. 3 shows lung sections from acute asthmatic mice administered with 10 μg of HK-X. Very few cells were associated with the airway (A) and (B) and mucus was limited to the surface of airway epithelial cell layer (C).

FIG. 4 shows lung sections from acute asthmatic mice administered with 1 μg of HK-X. Therapeutic effect dimished, with an increase in cellular infiltrates (A), and increase in mucus secretion into airways (B) and (C).

FIG. 5 shows lung sections from OVA-immunized mice challenged with either saline (A) or vehicle (0.05% DMSO) (B). No mucus secretion was detected in the airways (C).

FIG. 6 is a schematic illustration of the immunization and treatment regime used in establishing a chronic asthma mouse model.

FIG. 7 is a histogram illustrating the granuloma number in lungs of chronic asthmatic mice.

FIG. 8 shows the histology of chronic asthmatic lung tissues from mice immunized weekly with OVA for 6 months and treated with either HK-X or saline. (A) shows lung histology of control mice, (B) shows histology of HK-X-treated mice, and (C) shows histology of OVA-challenged but untreated mice.

FIG. 9 shows light micrographs of chronic asthmatic mouse lung tissue accumulation of collagen fibrils. (A) shows a lung section of a control mouse administered with saline, (B) shows a lung section of a mouse treated with HK-X, and (C) shows a lung section of an OVA-immunized but untreated mouse.

FIG. 10 shows lung sections of mice chronically OVA-immunized and treated with saline.

FIG. 11 shows lung sections of mice chronically OVA-immunized and treated with vehicle (0.5% DMSO).

FIG. 12 is a histogram illustrating the histomorphometry in chronic asthma.

FIG. 13 is a histogram illustrating the frequency of mucus containing cells in the airways of chronic asthmatic mice after various treatments.

FIG. 14 is a histogram illustrating the effects of various treatments on eosinophil and neutrophil infiltrates in the lungs of chronic asthmatic mice.

FIG. 15 is a schematic illustration of the immunization and treatment protocol with HK-X and dexamethasone in an acute asthmatic mouse model.

FIG. 16 is a histogram comparing the effects of intranasal administration of dexamethasone and HK-X on OVA-specific IgE levels.

FIG. 17 is a schematic illustration of the immunization and treatment protocol with HK-X and a control peptide in an acute asthmatic mouse model.

DETAILED DESCRIPTION OF THE INVENTION

In accord with the present invention, certain small peptides having the formula f-Met-Leu-X where X is selected from the group consisting of Tyr, Tyr-Phe, Phe-Phe and Phe-Tyr have been found to have surprising activity for downregulating the levels of IgE. As a result, such peptides are useful for treatment of a variety of indications resulting from IgE mediated responses. The peptides of the present invention can be used to replace corticosteroids in any application in which corticosteroids are used.

Preferred peptides, in accord with the present invention, reduce blood IgE levels and block IgE activation of lymphocytes such as, for example, macrophages, monocytes, eosinophils, neutrophils, TNF, and the like.

Continued mast cell degranulation and its release of leukotrienes, histamines, and other cytokines also decrease, or cease entirely in preferred embodiments, following treatment with peptides of the present invention. In accord with preferred embodiments of the present invention, the peptides also can reduce the infiltration of eosinophils, basophils and neutrophils into inflammatory tissues. Lymphocytes, eosinophils, and neutrophils do not exhibit chemotaxis in response to preferred peptides of the present invention. As a consequence, the chemotactic adhesion, migration and

aggregation of lymphocytes, eosinophils and neutrophils to the site of inflammation is significantly reduced, as is vascular permeability at the inflammation site. Further, preferred compounds of the present invention exhibit no toxicity to vital organs such as heart, liver and lungs.

The peptides of this invention can be prepared by conventional small peptide chemistry techniques. The peptides when used for administration are prepared under aseptic conditions with a pharmaceutically acceptable carrier or diluent.

The pharmaceutical compositions may conveniently be presented in unit dosage form and prepared for each type of indication resulting from IgE-mediated responses that is to be treated. The compositions may be prepared by any of the methods well known in the art of pharmacy. Methods typically include the step of bringing the active ingredients of the invention into association with a carrier that constitutes one or more accessory ingredients.

For example, doses of the pharmaceutical compositions will vary depending upon the subject, type of indication to be treated, and upon the particular route of administration used. Dosages of active peptide when treating acute IgE-mediated responses can range from 0.1 to 100,000 μg/kg a day, more preferably 1 to 10,000 μg/kg. Most preferred dosages range from about 1 to 100 μg/kg of body weight, more preferably from about 1 to 20 μg/kg and most preferably 10 to 20 μg/kg. Dosages of active peptide when treating chronic IgE-mediated responses can range from 0.1 to 100,000 μg/kg a day, more preferably 1 to 10,000 μg/kg. Most preferred dosages range from about 1 to 1000 μg/kg of body weight, more preferably from about 1 to 100 μg/kg and most preferably 50-70 μg/kg. Doses are typically administered from once a day to every 4-6 hours depending on the

severity of the condition. For acute conditions, it is preferred to administer the peptide every 4-6 hours. For maintenance, it may be preferred to administer only once or twice a day. Preferably, from about 0.18 to about 16 mg of peptide are administered per day, depending upon the route of administration and the severity of the condition. Desired time intervals for delivery of multiple doses of a particular composition can be determined by one of ordinary skill in the art employing no more than routine experimentation.

Routes of administration include oral, parenteral, rectal, intravaginal, topical, nasal, ophthalmic, direct injection, etc. In a preferred embodiment, the peptides of this invention are administered to the patient in an IgE downregulating effective amount. An exemplary pharmaceutical composition is an IgE modulating effective amount of a peptide in accord with the present invention that provides an IgE downregulating effect, typically included in a pharmaceutically acceptable carrier.

The term "pharmaceutically acceptable carrier" as used herein, and described more fully below, includes one or more compatible solid or liquid filler diluents or encapsulating substances that are suitable for administration to a human or other animal. In the present invention, the term "carrier" thus denotes an organic or inorganic ingredient, natural or synthetic, with which the molecules of the invention are combined to facilitate application. The term "IgE modulating-effective amount" is that amount of the present pharmaceutical compositions, which produces an IgE downregulating effect on the particular condition being treated. Various concentrations may be used in preparing compositions incorporating the same ingredient to provide for variations in the age of the patient to be treated, the severity of the condition, the duration of the treatment and the mode of administration.

The carrier must also be compatible. The term "compatible", as used herein, means that the components of the pharmaceutical compositions are capable of being commingled with a small peptides of the present invention, and with each other, in a manner such that does not substantially impair the desired pharmaceutical efficacy.

The small peptides of the invention are typically administered *per se* (neat). However, they may be administered in the form of a pharmaceutically acceptable salt. Such pharmaceutically acceptable salts include, but are not limited to, those prepared from the following acids: hydrochloric, hydrobromic, sulfuric, nitric, phosphoric, maleic, acetic, salicylic, p-toluene-sulfonic, tartaric, citric, methanesulphonic, formic, malonic, succinic, naphthalene-2-sulfonic, and benzenesulphonic. Also, pharmaceutically acceptable salts can be prepared as alkaline metal or alkaline earth salts, such as sodium, potassium or calcium salts of the carboxylic acid group. Thus, the present invention provides pharmaceutical compositions, for medical use, which comprise peptides of the invention together with one or more pharmaceutically acceptable carriers thereof and optionally any other therapeutic ingredients.

The compositions include those suitable for oral, rectal, intravaginal, topical, nasal, ophthalmic or parenteral administration, all of which may be used as routes of administration using the materials of the present invention. Pharmaceutical compositions containing peptides of the present invention may also contain one or more pharmaceutically acceptable carriers, which may include excipients such as stabilizers (to promote long term storage), emulsifiers, binding agents, thickening agents, salts, preservatives, solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is

incompatible with the peptide of this invention, its use in pharmaceutical preparations is contemplated herein. Supplementary active ingredients can also be incorporated into the compositions of the present invention.

Compositions suitable for oral administration are typically prepared as an inhalation aerosol, nebule, syrup or tablet. Compositions suitable for topical administration typically are prepared as a cream, an ointment, or a solution. For treating an acute IgE-mediated response, the concentrations of the peptide active ingredient in such compositions is typically less than 1000 μg/ml, more preferable less than 500 μg/ml, and most preferably from about 200 to 400 μg/ml. For treating a chronic IgE mediated response, the concentrations of the peptide active ingredient in such compositions is typically less than 3 mg/ml, more preferable less than 2 mg/ml, and most preferably from about 1 to 1.5 mg/ml.

Compositions of the present invention suitable for inhalation administration may be presented, for example, as aerosols or inhalation solutions. An example of a typical aerosol composition for treating acute IgE-mediated responses consists of about 0.1 to 100 μg of microcrystalline peptide suspended in a mixture of trichloro-monofluoromethane and dichlorodifluoromethane plus oleic acid, per dose. A more preferable amount of microcrystalline peptide in the composition is 1 to 50 μg, and most preferable is 10 to 20 μg per dose of the aerosol composition. An example of a typical aerosol composition for treating chronic IgE-mediated responses consists of about 0.1 to 1000 μg of microcrystalline peptide suspended in a mixture of trichloro-monofluoromethane and dichlorodifluoromethane plus oleic acid, per dose. A more preferable amount of microcrystalline peptide in the composition is 1 to 100 μg, and most preferable is 50 to 70 μg per dose of the aerosol composition. An example of a typical solution consists of the desired quantity of peptide

dissolved or suspended in sterile saline (optionally about 5 % v/v dimethylsulfoxide ("DMSO") for solubility), benzalkonium chloride, and sulfuric acid (to adjust pH).

Compositions of the present invention suitable for oral administration also may be presented as discrete units such as capsules, cachets, tablets or lozenges, each containing a predetermined amount of the peptide of the invention depending on the type of IgE mediated response to be treated, or which may be contained in liposomes or as a suspension in an aqueous liquor or non-aqueous liquid such as a syrup, an elixir, or an emulsion. An example of a tablet formulation base includes corn starch, lactose and magnesium stearate as inactive ingredients. An example of a syrup formulation base includes citric acid, coloring dye, flavoring agent, hydroxypropylmethylcellulose, saccharin, sodium benzoate, sodium citrate and purified water.

Compositions suitable for parenteral administration conveniently comprise a sterile aqueous preparation of the molecule of the invention, which is preferably isotonic with the blood of the recipient. This aqueous preparation may be formulated according to known methods using those suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example as a solution in 1,3-butane diol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution and isotonic sodium chloride solution. In aqueous solutions, up to about 10 % v/v DMSO or Trappsol can be used to maintain solubility of some peptides. Also, sterile, fixed oils may be conventionally employed as a solvent or suspending medium. For this purpose, a number of fixed oils can be employed including synthetic mono- or diglycerides. In addition, fatty acids (such as oleic acid or neutral fatty acids) can be used in the preparation of

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A protocol for administration of ovalbumin (OVA) as a model allergen has been developed to induce acute allergen-specific pulmonary disease in normal Balb/C mice. The protocol involves intraperitoneal (i.p.) immunization of mice with 100 μg of ovalbumin (OVA) in alum adjuvant on days 1 and 14, and single intranasal (i.n.) doses of 50 to 100 μg of OVA in normal saline on days 14, 25, 26, and 27. Control mice receive alum alone by i.p. injections, and normal saline alone by i.n. administrations. On day 28, OVA-immunized mice display a disease strikingly similar to allergen-induced human asthma. This animal model has been used for the evaluation of drug efficacy in allergic acute pulmonary disease.

The Mouse Model for Late-Phase Chronic Allergen-Specific Pulmonary Disease

The protocol for administration of ovalbumin (OVA) as a model allergen to induce late-phase chronic allergen-specific pulmonary disease in normal Balb/C mice includes intraperitoneal (i.p.) immunization of mice with 100 μg of ovalbumin (OVA) in alum adjuvant on days I and 14, and single intranasal (i.n.) doses of 50 to 100 μg of OVA in normal saline on days 14, 25, 26, and 27 and then weekly thereafter for up to 6 months. Control mice receive alum alone by i.p. injections, and normal saline alone by i.n. administrations. On day 28, OVA-immunized mice display a disease strikingly similar to allergen-induced human asthma. This animal model is also useful for the evaluation of drug efficacy in chronic allergic pulmonary disease.

Materials and Methods

Special Reagents: Crystalline OVA was obtained from Pierce Chem. Co. (Rockford, IL) and aluminum potassium sulfate (alum) from Sigma Chemical, St. Louis, MO. The OVA (500ug/ml) was mixed with equal volumes of 10% (wt/vol.) alum in distilled water. The mixture was adjusted to pH 6.5 with 10 N NaOH and incubated for 60 min at room temperature.

The material was centrifuged at 750 g for 5 min; the pellet was resuspended to the original volume in distilled water and used within 1 hr.

Immunization Protocol: the immunization protocol consisted of intraperitoneal administration of 100 µg OVA in alum on day 1 followed by intraperitoneal administration of 100 µg OVA in alum combined with intranasal administration of 100 µg OVA in saline on day 14. On days 25, 26, and 27 the mice were challenged with intranasal OVA (100 µg in saline). For acute asthmatic studies, the animals were euthanized on day 28 and lungs removed. For chronic asthmatic studies, mice were immunized weekly thereafter for up to 6 months.

Analyses

ELISA protocol for serum IgE: Immulon 2 Microtiter plates (Dynex Technologies) were coated with OVA solution in 50 mM carb-bicarbonate buffer, pH 9.6 at 4 C overnight and blocked with 0.1% casein for 2 hr at room temperature (RT). All test sera were diluted 1:100 in Tris-NaCL buffer, pH 8.0 containing 0.1 % casein prior to incubation with OVA coated plates. A positive serum sample known to contain IgE antibodies to OVA and normal serum samples from unimmunized mice were included in each assay as controls. The serum samples were incubated on the plates at room temperature for 2 hours and washed 6X with PBS. Appropriately diluted secondary antibody (sheep, antimouse IgE Biotin (Binding Site cat # PB 284, lot # 026917) was added for 2 hr at RT and the plates were washed 6X with PBS. OPD, Urea and peroxide solution were added for 30 min at RT. The reaction was stopped with 2.5 M sulfuric acid. OD was read at 490/630 nm. All samples were run in duplicate. Inter and intra sample variation of positive controls was less than 10% of the means.

Lung Histology: The lung and trachea were removed and fixed in 10% neutral buffered formalin. The tissues were embedded in paraffin and cut

into 7 μm sections. After deparaffinization and hydration, the sections were stained with eosinophil staining solution and counterstained with methylene blue. Alcian blue, toluidine blue, and periodic acid Schiff stains identified mucus within the airway. Tissues were examined by light microscopy.

Bronchoalveolar Lavage (BAL): The left lung was tied off at the mainstem bronchus. The right lung was ravaged with 0.4 ml of normal saline three times, and the fluid pooled. The total cell number was determined using a hemocytometer. The remaining cells were pelleted by centrifugation and the cells placed into a 10% BSA solution and resuspended. The cells were placed on a microscope slide and stained with an eosinophil staining solution (eosin with methylene blue counterstain).

Histomorphometric Analysis of Lung: The following parameters of allergic pulmonary disease were measured in the experiments reported here:

1. Airway plug scores were scored as previously reported (Henderson et al. *J. Exp. Med.* **184**:1483-1494, 1996). A scoring system from + to ++++ was used, reflecting the degree of severity of mucus secretion.

2. Total mucous cells were estimated by randomly counting the number of epithelial cells containing mucus per 100 epithelial cells in medium to large airways (600 μm to 1,000 μm diameters). Ten fields were counted in different lung lobes.

3. Cell density of infiltrating cells located either in the perivascular compartment or in the areas adjacent to airways (neutrophils, eosinophils, monocytes and lymphocytes) was approximated by using a scoring system ranging from o to ++++. A score of + indicates an inflammatory cell layer of 3 but less than 5 cells; ++ indicates an inflammatory density of 5 cells to10 cells; +++ indicates an inflammatory density of 10 to 20 cells; and ++++ indicates an inflammatory density of 20 to 40 cells.

4. Numbers of various cell types were quantified by counting the numbers per high power held (1 0X by 40X).

5. Degree of edema was calculated by using a scoring system wherein the degree of accumulation of fluid surrounding blood vessels was estimated.

Statistical Analyses of Histomorohometric Data: SigmaStat version 2.0 was used to perform statistical analyses. Differences were analyzed for significance ($p<0.05$) by ANOVA using the appropriate posthoc tests for independent means. SigmaPlot version 4.0 or GraphPad Prism was employed for the construction of graphical representations of the data.

EXAMPLE 1: Therapeutic Dose Response of Acute Asthmatic Mice to HK-X

Ideally, an experiment which demonstrates that therapeutic efficacy correlates with drug dosage will show three distinct regions of behavior: 1) At low doses, there will be no therapeutic effect; 2) At higher dosage, therapeutic efficacy will be dose dependent; 3) The third range of doses (highest) will not demonstrate therapeutic efficacy greater than that observed at the highest middle range dose.

Dose response curves are an important source of information on dosages safe for human use. Occasionally, when doses of drugs that exceed the optimal therapeutic dosages are administered, toxic responses can be observed. This is particularly true if the drug is administered in situ, such as intranasally.

To establish therapeutic effectiveness of a range of doses of f-met-Leu-Phe-Phe (HK-X) during the acute effector phase of bronchial asthma at days 25, 26 and 27 induced by repeated immunization with OVA, doses of 0.1, 1.0, 10 and 50 µg of intranasal HK-X were chosen. HK-X was

administered 30 min before OVA challenge. Control groups consisted of OVA-immunized and OVA-challenged mice as well as animals immunized with Alum in saline and challenged with saline alone. All animals were sacrificed one day after (day 28) the final OVA challenge. Serum IgE levels were determined and serum and lung tissues were collected for further analysis.

To first establish the optimal dose that will effectively downregulate serum IgE levels in an acute asthma model, 0.1, 1.0, 10 and 50 μg doses of HK-X (in 40 μL of saline) were infused into the lung 15-30 min prior to antigenic challenge on days 25, 26, and 27. A dose response curve of serum IgE levels is depicted in Figure 1.

The effects of varying doses of HK-X on the response of lung tissue to acute allergic challenge are depicted in Figures 2A to 4C. Fifty micrograms of HK-X administered intranasally to acute asthmatic mice provided some degree of protection against the effects of acute asthma (Figs. 2A-2C). There was limited perivascular and peri-bronchial accumulation of inflammatory cells (Figs. 2A and 2B). Figure 2C demonstrates that mucus accumulation was present but limited.

Ten micrograms of HK-X appeared to be the most efficacious dose (Figs. 3A-3C). Figures 3A and 3B show minimal inflammatory infiltrate surrounding vessels and airways. The degree of mucus secretion in airways is illustrated in Figure 3C. The mucus is confined to the surface of the airway epithelial cells.

As the dose of HK-X decreased 10 fold to 1 μg, therapeutic effect diminished. The amount of perivascular and airway inflammation increased (Figure 4A). There was a corresponding increase in mucus secretion by airway epithelial cells (Figure 4 B and 4C).

For the purposes of contrast and control, Figures 5A through C illustrate the benign response of immunized mice to administration of saline or the HK-X vehicle (0.05% DMSO). As shown in Figures 5A and 5B, there was little detectable inflammatory infiltrate in the perivascular or periairway zones of the lung. Correspondingly, there was no accumulation of mucus in the airway lumen or on the airway epithelial cell surfaces (Figure 5C).

Of the key histological measurements of the severity of acute asthma, mucus plug, the numbers of eosinophils and the fraction of airway cells secreting mucus showed a dose dependent improvement after treatment with 0.1 μg to 10 μg HK-X. 10 μg of HK-X provided a 70% reduction in mucus plug score ($p<0.05$). Interestingly, 50 μg of HK-X provided significantly less reduction in mucus plug ($p<0.05$). This same pattern of responsiveness was observed for the numbers of eosinophils and fraction of airway cells secreting mucus. The 10 μg dose of HK-X showed a 57% decline in the number of interstitial eosinophils, which was significantly greater than the 0.1 μg dose effect of ($p<0.05$). The reduction in eosinophils by the 50 μg dose was less than one-half that provided by 10 μg HK-X ($p<0.05$).

The fraction of airway cells secreting mucus was also inhibited in a dose dependent manner from 0.1 μg to 10 μg (37% reduction, $p<0.05$). The 50 μg dose provided a small amount of reduction (11%) which was not significantly less than the 0.1 μg dose of HK-X. The effect of HK-X on the accumulation of fluid surrounding vessels showed a modest decline at 10 and 50 μg doses. However, none of the doses was different from the 0.1 μg dose of HK-X. The dose of HK-X showing the greatest reduction in the inflammatory cell score or accumulation of inflammatory cells was 10 μg.

These data demonstrate that the following parameters showed a dose response effect: serum IgE levels and histopathological features (cellular infiltration, mucus plug formation, and total eosinophlis in interstitium). Ten micrograms of HK-X administered intranasally was the most effective dosage compared to lower doses and a higher dose, 50 μg. Compared to controls, animals treated with 10 μg of HK-X demonstrated a 60% reduction in serum IgE levels, 50% reduction in cellular infiltration of the lung, 70% reduction in mucus plug formation and 67% reduction in eosinophil number.

EXAMPLE 2: Chronic Asthma With and Without HK-X Intervention

The animal model is also useful for the evaluation of drug efficacy in chronic allergic pulmonary disease. In this study, an immunization period of 6 months induced a persistent inflammation that was maintained by weekly intranasal challenges with OVA. The mice were treated with saline 8 times over a 20 day period to assess changes which occurred in the lungs. HK-X treatments of mice with chronic asthma were performed as indicated in Figure 6. 50 μg of HK-X (in 50 μL of saline containing less than 2.5% DMSO) was administered i.n. for a total of 8 dosages delivered over a period of 16 days. The animals were sacrificed 4 days after the last saline or HK-X dose. The experimental results were compared between HK-X treated and HK-X untreated mice.

IgE levels of antibody to OVA in the blood of mice challenged with or without OVA are shown in Table 1. It is important to note that all animals were OVA immunized for the first 6 months, however, the group denoted as "saline" were administered saline intranasally but not challenged with OVA during the terminal 20 day period. Therefore, these IgE levels were carried over from the immunization period and were used as background values from which all comparisons were corrected. For example, animals treated

with either saline or DMSO and OVA challenged had a 36% increase in IgE levels compared to a 14% increase in IgE levels in the animals treated with HK-X and OVA challenged. The amount of suppression of IgE levels by HK-X was calculated to be ~60%.

TABLE 1: IgE Values in the Sera of Chronic Asthmatic Mice

TREATMENT	MEAN OD	∀SE	P VALUE
DMSO/OVA	1.115	∀0.017	@0.111
SALINE/OVA	1.113	∀0.093	@0.111
HK-X/OVA	0.929	∀0.033	0.049 @ 0.111
SALINE	0.814	∀0.079	@0.111

Note: These values represent relative IgE levels as OD values from the ELISA test.

One of the important characteristics of chronic asthma in the murine model is the appearance of granulomatous structures in the lung. The effective IgE downregulating dose of 50 μg of HK-X significantly ($p<0.05$) reduced the numbers and sizes of these structures in the lungs of treated animals compared to animals permitted to spontaneously reduce collagen deposition [Saline or DMSO] (Fig. 7).

Furthermore, during the immunization of mice with OVA and the subsequent treatment with HK-X at a dosage of 50 μg for a total of 8 times over a 20 day period, no adverse reactions or signs of sickness were observed. The mice were active during the experimental period. Examination of the lung tissues from groups of animals immunized with only OVA revealed severe pulmonary pathological changes consistent with characteristics of chronic asthma observed in humans. There was a significant infiltration of inflammatory cells in association with outer

boundaries of the airway basal lamina (interstitial regions) and blood vessels (Fig. 8C). When animals were treated with 8 doses of 50 μg of HK-X per treatment over a 20-day period, the number of inflammatory cells were clearly reduced around airways and blood vessels (Fig. 8B). Saline inhalation by saline sham or control immunized mice resulted in patent airways and blood vessels with normal appearance (Fig. 8A). OVA immunized mice contained increased accumulations of collagen (blue color) around vessels and airways (Figure 9C). However, lungs treated with HK-X demonstrated a reduced level of collagen deposition (Figure 9B). In control mice (administered HK-X in saline) the pulmonary tissue was free of inflammatory cells and fibrotic collagen deposits (Figure 9A). In contrast, in OVA sensitized mice and treated only with either saline (Figures 10A-10C) or 0.5% solution DMSO (Figures 11A-11C), airways contained mucus and had mucus secreting cells when Alcian blue at pH 2.3 was used to visualize mucus. The amount of perivascular and airway inflammatory cell infiltration was similar. Greater than 60 percent of the airways of these 6 month-old chronic asthmatic mice were plugged with mucus (Figs. 10A-10B and 11A-11B, respectively).

The pulmonary tissue changes in chronic asthma were also assessed by morphometry methods to quantify the degree of persistent inflammation, deposition of fibrotic collagen and airway narrowing with structural changes. In Figures 12 through 14, the responses of animals immunized with OVA for 6 months and treated with various agents are shown in the left panels of the figures, whereas animals sham immunized and treated with saline are shown in the right panel. There were significant differences ($p < 0.05$) between the degree of mucus plug formation in HK-X treated animals versus animals given only saline for the 20 day period (Fig. 12). The animals insumated with the vehicle (0.5 % DMSO) for H K-X compared to HK-X also did not demonstrate any improvement in the mucus plug score. The results were the same for saline treatments. The patterns of

responses for inflammatory cell accumulation in and about the airways paralleled the mucus plug data for the various experimental treatments (Fig. 12). When given 8 doses of 50 μg each of HK-X in 40 μL vehicle intranasally over a 1 6-day period, there was a significant reduction in mucus accumulation and mucus cells occurrence in the airway (Fig. 13). Again the saline treatment alone demonstrated no therapeutic effects but HK-X did significantly reduce the number of mucus containing cells within the airways ($p<0.05$). This observation further substantiates that there was no spontaneous repair after establishment of OVA-induced chronic asthma. In the analyses of the numbers of infiltrating inflammatory cells in association with airway, the data showed that eosinophils and neutrophils per unit area was also reduced (Fig. 14). Intranasal saline infused animals maintained high levels of eosinophils as did DMSO treated animals ($p>0.05$); however, HK-X treatment reduced the numbers of eosinophils significantly compared to the two control groups ($p<0.05$).

These studies show that there was very little or no spontaneous reduction in airway inflammation or of mucus cell secretion in this model of allergen induced chronic asthma unless HK-X was administered. In this model, mice were sensitized to OVA and exposed to OVA via intranasal route weekly for 5 months and were treated with HK-X intranasally 8 times over a 20 day period. This allergen immunization and challenge regimen led to a chronic airway infiltration of eosinophils and other types of inflammatory cells, accumulation of mucus in the airways and hyperplasia of mucus secreting cells. Administration of an effective IgE-downregulating dose of 50 μg of HK-X reduced airway hypersecretion, hyperplasia of mucus cells, and recruitment of eosinophils and neutrophils. These results indicate that by administering an efffective amount of HK-X and downregulating IgE levels, HK-X can also downregulate IgE-mediated responses such as airway hypersecretion of mucus and the deposition of collagen that occur in this allergen-induced model of asthma.

EXAMPLE 3: Effects of HK-X and Dexamethasone in Acute Murine Asthma

Glucocorticoids are potent inhibitors of inflammatory mediators produced by a variety of cell types, including T cells, mast cells, monocytes, dendritic cells and eosinophils. Glucocorticoids are effective in the treatment of human asthma when inhaled or used systemically. They suppress inflammatory cell infiltration and have been demonstrated to decrease mucus secretion and pulmonary edema. These responses relate to direct effects of glucocorticoids on bronchial epithelial cells. Equally important, steroids reduce bronchial hyperresponsiveness. Because of their demonstrated efficacy, the glucocorticosteroids represent a mainline therapeutic armamentarium in the treatment of asthma and could be used without reservation but for well documented cumulative toxicity that limits their value over time. Because of their value as the current standard of efficacy for asthma, new compounds for asthma treatment should be evaluated in comparison to glucocorticoids.

This experiment compared the effectiveness of HK-X to dexamethasone, a widely used glucocorticoid, to modulate mucus release, eosinophil numbers, edema and allergen specific IgE levels in this mouse model. Comparable dosages of 10 μg and 50 μg of HK-X and dexamethasone were used in this study. Intranasal administration was used for both drugs at all doses. Fifty micrograms of HK-X was selected as the high dose based on the previous results from the chronic asthma model. An outline of the immunization and treatment protocol is shown in Figure 15.

The experimental parameters showed that 10 μg of intranasal HK-X was more effective than either 10 μg or 50 μg of dexamethasone in reducing serum IgE levels (Figure 16). Ten μg of HK-X reduced serum IgE levels by

28%. HK-X was also more effective than dexamethasone at improving two histopathological features: cellular infiltrate and total number of eosinophils in interstitium. Both 10 μg and 50 μg doses of HK-X significantly reduced inflammatory infiltrate by 54% compared to the OVA control ($p<0.05$) and were significantly more effective than 10 μg (13%) and 50 μg (13%) of dexamethasone ($p<0.05$). Both the 10 μg and 50 μg doses of HK-X were more effective than 10 μg of dexamethasone ($p<0.05$). Ten μg of HK-X decreased the eosinophil cell count by 57%, while the same dose of dexamethasone decreased the eosinophil cell count by 13%.

A high dose of 50 micrograms of HK-X administered intranasally was as effective as either dosage of dexamethasone in reducing the following histopathological features: number of eosinophils in Bronchoalveolar Lavage (BAL), mucus plug formation, percentage of airway mucus secreting cells, number of interstitial eosinophils, and edema. These results establish the comparative efficacy of HK-X and a glucocorticoid, dexamethasone, in the murine asthma model.

EXAMPLE 4: Effects of HK-X and a Related Control Peptide in Acute Murine Asthma

This experiment compared the effectiveness of HK-X to a related member of the peptide family, f-Met-Met (referred to as the control peptide) in relation to the following measures: mucus release, cellular infiltration, eosinophil numbers, edema and allergen specific IgE levels in the acute asthma mouse model. Comparable dosages of 50 μg of HK-X and the control peptide were used in this study. Intranasal administration was used for both compounds. The immunization and treatment regime is outlined in Figure 17.

While belonging to the same family of chemical compounds and being closely related in molecular size, the control peptide did not exhibit any of the therapeutic properties of HK-X. Most significantly, 50 μg of HK-X caused a 7% decrease in the serum IgE levels in the sera to the allergen, OVA ($p>0.05$). 50 μg of the control peptide did not affect the serum IgE levels ($p>0.05$). Furthermore, the control peptide delivered in vehicle and administered to control animals promoted clear-cut pro-inflammatory increases in the following parameters: mucus plug formation, number of airway cells secreting mucus, and the degree of interstitial inflammatory cells. In previous experiments, HK-X did not demonstrate any pro-inflammatory changes in the histological parameters measured. Therefore, the unique composition of HK-X appears responsible for its efficacy in downregulating IgE levels and IgE-mediated responses.

EXAMPLE 5: Pulmonary Tissue Response to Long Term Dosing of High Therapeutic Levels of HK-X

To determine whether there are potential toxic effects of long-term intranasal exposure to HK-X at the higher end of the therapeutic dose range, mice were exposed to weekly doses of 20 μg of intranasal HK-X for 3 months. During the last two weeks, the intranasal dose of HK-X was increased to 50 μg. Lung tissue was collected 24 hr after the last H K-X administration for histological analysis.

Weekly administration of 20 μg of HK-X intranasally for 3 months followed by 2 weeks of administration of 50 μg did not cause pathologic alterations of lung tissue. There was no difference ($p>0.05$) between saline and HK-X administration regarding mucus plug formation and inflammatory infiltrate. Secretion of mucus by airway cells was elevated after administration of HK-X but this is not judged to be biologically significant. A similar phenomenon was observed regarding the number of

interstitial eosinophils. While HK-X treated animals had approximately 2 eosinophils per 2,200 μ^2, the saline treated animals demonstrated less than 1 per unit area ($p<0.05$). Livers, spleens, and kidneys were examined for pathological changes. Except for occasional foci of inflammatory cells in the livers of animals from control and treated groups, no pathologic changes were observed. These data establish the tolerability of supra-therapeutic doses of HK-X in mice.

EXAMPLE 6: Immunogenicity and Antigenicity of HK-X

The objective of this study is to determine whether HK-X, when administered to mice via several different routes, will produce an immune response as assessed by antibody production. Thus, immunogenicity and antigenicity were both evaluated in relation to HK-X in this study.

HK-X is a small tetrapeptide. In most cases, such small molecules are poorly immunogenic; however, *in vivo*, small molecules may conjugate or absorb to (become haptens) larger proteins or to blood cells (carriers). Penicillin, quinidine and α-methyl dopa allergic responses are examples of such haptenic responses. Antibodies to the haptens can produce anemia and immune complex diseases because of the destruction of red cells (carriers).

A number of haptens such as dinitrophenol (DNP) or trinitrophenol (TNP) used experimentally are covalently linked to carrier molecules. The more antigenic the carrier molecule, the more likely that an immune response to the hapten will be elicited. Keyhole Limpet hemocyanin (KLH) is a widely used carrier and generally supports potent antibody responses to haptens like DNP or TNP.

The use of adjuvants greatly increases the likelihood that a potential immunogen will elicit an immune response. Complete Freunds Adjuvant (CFA) or bacterial peptidoglycans have been widely used to stimulate immune responses to poorly immunogenic haptens.

Therefore, after first determining availability of antibodies from normal drug exposure routes (with no anti- HK-X reactivity), the potential immunogenicity of HK-X was examined when it was coupled to KLH and administered in bacterial adjuvant. These extreme conditions determined whether HK-X could be immunogenic.

Materials and Methods

Immunogenic Conjugates of HK-X: HK-X was conjugated to KLH via a 12 to 20 carbon spacer added at the carboxy terminus. The linkage was completed through lysine residues on the KLH. United Biochemical, Seattle, WA, prepared the conjugates.

Preparation of Immunogen: HK-X-KLH conjugate suspended in PBS at 0.1 mg/ml was emulsified in complete Freund's adjuvant (CFA) containing 1.0 mg/ml bovine Mycobacterium tuberculosis at a 1:1 ratio.

Adjuvant Immunization Protocol: Balb/C female mice were immunized intradermally with 0.1 ml emulsion, boosted 4 weeks later and bled at 6 weeks.

Soluble Immunization Protocol: Balb/C female mice were injected intraperitoneally with 100 µg of the conjugate without adjuvant in a volume of 0.1 ml to 0.2 ml. The mice were bled after 21 days.

Normal Drug Exposure Routes: Sera were collected from animals administered HK-X via the intranasal route in therapeutic asthma studies.

Determination of antibodies: ELISA analyzed antibodies to conjugated and unconjugated HK-X. Immulon 2 Microtiter Plates (Dynex Technologies cat. # 3455) were coated overnight at 4°C with the following HK-X or HK-X conjugates at 10 μg/ml in PBS:

- HK-X- peptide alone
- HK-X - KLH - peptide conjugated to KLH
- HK-X LISA- peptide conjugated to BSA
- HK-X - Spacer- peptide with 12 carbon linear spacer.

Wells were washed the following day with PBS and then blocked for 30 minutes at room temperature with sample dilution buffer consisting of 0.1 M Tris - 0.15M NaCl buffer, pH 8.0, and 0.1% casein (ICN cat # 902896, lot 99333). Mouse sera samples were diluted either 1:100 or 1:200 with the same buffer, added to the wells and incubated 2 hours at room temp. Wells were then washed with PBS and incubated with goat anti-mouse IgG peroxidase conjugated secondary antibody (Cappel cat # 55554, lot # 39714) for 2 hours at room temp. After washing with PBS, wells were reacted with OPD chromagen (SIGMA cat # P-9187, lot 18H82111) for 30 minutes at room temp. The reaction was stopped with 50 μl of 2.5 M sulfuric acid. The ODs were then determined using a BIO-TEK EL800 reader at 490/630.

Results

Determination of HK-X from normal, drug exposure route: Sera from the following groups of mice were tested for anti-HK-X reactivity: OVA-induced asthma and HK-X treated, OVA-induced asthma and DMSO (vehicle) treated control, saline-immunized and DMSO (vehicle) treated. Mice were treated intranasally every other day with 50 μg of HK-X or vehicle for 16 days.

No IgG reactivity was observed to HK-X conjugated to either the 12-C spacer (HK-X+Spacer), KLH (KLH-HK-X) or BSA (BSA-HK-X). IgG reactivity to OVA was observed in all OVA-immunized mice and one saline-immunized control mouse and served as a control for the ELISA. IgG reactivity to KLH-HK-X and BSA-HK-X was observed in sera from animals immunized with KLH-HK-X in adjuvant and served as a control for the coating of these antigens onto the ELISA plate.

Soluble-immunized HK-X coupled to a carrier. Mice were immunized with soluble KLH-HK-X and bled after 21 days. The results of the ELISA show that 4/5 serum samples reacted to KLH and KLH-HK-X but no reactivity to BSA-HK-X or HK-X +spacer was observed indicating that antibodies were not generated against the HK-X that was coupled to KLH after immunization with soluble carrier coupled to HK-X.

Adjuvant-immunized HK-X coupled to a carrier. To force the generation of antibodies to HK-X, mice were immunized with KLH or KLH-HK-X in complete Freund's adjuvant, boosted once and bled after 6 weeks. The results of the ELISA show that antibodies were generated against KLH. Antibodies were also generated to HK-X. This was supported by the following: 1) antibody reactivity to KLH-HK-X from KLH-HK-X sera was 2 fold higher than from KLH only immune sera and 2) sera from KLH-HK-X immunized HK-X immunized mice reacted to BSA-HK-X but not to BSA alone. However, no antibody reactivity was observed to H K-X coupled to the 12C spacer.

From the results of these studies, several conclusions about the immunogenicity and antigenicity of the HK-X peptide can be made. First, mice did not generate antibodies to HK-X after therapeutic intranasal administration of the peptide for 16 days. Second, mice did not generate

antibodies when immunized with soluble peptide conjugated to the immunogenic carrier KLH. Third, mice can be forced under extreme conditions to generate antibodies to HK-X when coupled to KLH and immunized with complete adjuvant. However, even in this case, antibody reactivity is probably generated to neo-epitopes created by the conjugation of HK-X and KLH since no antibody reactivity could be detected to HK-X conjugated to the 12C spacer. This conclusion is supported by the observation that addition of free HK-X to the antiserum for at least 30 min prior to incubation with the test antigen, HK-X-KLH, did not reduce antibody reactivity to HK-X-KLH.

Thus, it appears unlikely that clinically relevant antibody or other immune responses to HK-X will be elicited in the clinical environment. There are five observations supporting such a notion. These observations are:

1) HK-X is only four amino acids in size (less than 600 Dalton), which makes it unlikely to become immunogenic;

2) All of the amino acids in HK-X are hydrophobic, whose property is not associated with immunogenicity;

3) To become immunogenic, HK-X would have to become covalently or electrostatically associated with a larger and immunogenic carrier in vivo;

4) Antibodies produced to HK-X are likely to be directed towards an epitope formed by the combination of the carrier and HK-X (neo-antigen);

5) Antibodies directed towards the neo-antigen react only weakly (low affinity) if at all to free HK-X.

EXAMPLE 7: Primate Toxicology Study of HK-X

This study was conducted at BIOSUPPORT, an animal research facility in Redmond, Washington, according to GLP standards. Six adult male and female macaque monkeys obtained from Charles River were

studied. Group A, considered a control group, consisted of two animals given vehicle (buffered saline with 3% DMSO) IV daily for five days. Blood sampling for CBC and chemistries was performed on days 0-4 and 7. Group B consisted of three animals dosed with 20 μg/kg of HK-X in vehicle (buffered saline with 3% DMSO) IV daily for five days. Blood sampling for CBC and chemistries was performed on days 0 – 4 and 7. Group C consisted of the three additional animals dosed with 150 μg/kg IV daily in an identical regimen. Group D consisted of all six animals from Groups B and C, dosed with 1000 μg/kg IV daily using the same regimen, five days after Group C animals completed their regimen. All animals were observed daily throughout the study for recording of weight and general health and behavior. At the end of the Group D regimen, all animals were euthanized, underwent necropsy, and had representative tissue samples from the following organs collected for histological analysis: liver, kidney, spleen, lung, heart, lymph node, and brain. Histopathological evaluation was performed by a board certified veterinary pathologist associated with BIOSUPPORT and independently by a histopathologist associated with Histatek.

These dosages of HK-X were selected based on the effective therapeutic dosages of HK-X of 10 and 50 μg/kg in the mouse asthma model.

No significant abnormalities of white blood cell, hematocrit/hemoglobin, or platelet counts were noted on any day or at any dose level. Similarly, no significant abnormalities of chemistry values were noted at any of the three dosing levels. No histological abnormality was noted in representative tissue samples of spleen, and lymph nodes obtained from animals who were exposed to either 20 or 150 μg/kg of HK-X followed by 1000 μg/kg daily, or in the vehicle control group. Minimal multifocal

lymphocytic infiltrate was noted in liver, kidney, heart, and lung tissue samples from both treated and control animals and was therefore judged unrelated to treatment. Mild glomerular lesions, common in aging macaques, did not segregate according to treatment and were thus also considered unrelated to treatment. Other minor histological changes were not considered significant.

There was no discernible toxicity observed in blood counts or chemistries obtained from six macaque monkeys exposed to dosage levels of HK-X significantly higher than dosages considered therapeutic. Minor histopathological changes noted in liver, kidney, spleen, lymph nodes, heart, and lung did not segregate according to treatment and were considered manifestations of background pathology or artifactual change related to euthanasia.

This primate study suggests that therapeutic amounts of HK-X can be useful in human treatment without apparent toxicity or side effects.

The invention has been described in detail with reference to preferred embodiments thereof. However, it will be appreciated that, upon consideration of the present specification and drawings, those skilled in the art may make modifications and improvements within the spirit and scope of this invention as defined by the claims.